A novel E-subgroup pentatricopeptide repeat protein DEK55 is responsible for RNA editing at multiple sites and splicing of *nad1* and *nad4* in maize

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Abstract

Background: Pentatricopeptide repeat (PPR) proteins form a large protein family that participates in RNA processing in organelles and plant growth. Previous reports regard E-subgroup PPR proteins as editing factors for RNA editing. However, additional functions and roles of the E-subgroup PPR proteins remain to be investigated.

Results: In this study, we developed and identified a new maize kernel mutant with arrested embryo and endosperm development, i.e., *defective kernel 55* (*dek55*). Genetic and molecular evidence suggested that the defective kernels was a result of a mononucleotide alteration (C to T) at +449 bp in the open reading frame (ORF) of Zm00001d014471 (hereafter referred to as *DEK55*). *DEK55* encodes an E-subgroup PPR protein within mitochondria. Molecular analyses showed that the editing ratio of 24 RNA editing sites was decreased and that of seven RNA editing sites was increased in *dek55* mutant kernels, which were distributed in 14 mitochondrial gene transcripts. Meanwhile, the splicing efficiency of the *nad1* introns 1 and 4 and *nad4* intron 1 was significantly decreased in *dek55* compared with that of wild-type (WT). These results indicate that DEK55 plays a crucial role in RNA editing at multiple sites as well as in the splicing of *nad1* and *nad4* introns. Mutation in the *DEK55* gene led to the dysfunction of mitochondrial complex I. Yeast two-hybrid assays showed that the DEK55 interacts with two multiple organellar RNA editing factors (MORFs), i.e., ZmMORF1 (Zm00001d049043) and ZmMORF8 (Zm00001d048291), respectively.

Conclusions: Our results demonstrated that a mutation in the *DEK55* gene affects the mitochondrial function essential for maize kernel development. Our results also provide novel insight into the molecular functions of the E-subgroup PPR proteins in plant organellar RNA processing.

Background

Pentatricopeptide repeat (PPR) proteins form a large protein family found in most land plants, with over 450 members identified in *Arabidopsis thaliana*, *Oryza sativa,* and *Zea mays* [1-5]. These proteins include standard tandem degenerate repeat motifs, which form a helix-loop-helix structure of approximately 35 amino acids. PPR proteins are mainly classified into P- and PLS-type subfamilies according to their PPR repeat motifs [2, 6, 7]. P subfamily PPR proteins contain only classical “P” motif repeats in tandem, while PLS subfamily PPR proteins consist of alternate repeats of three PPR motifs of different lengths. These are usually divided into PLS, E, E+, and DYW subgroups based on the presence of E, E+, or DYW domains at the carboxy-terminal end [2]. A new class of PPR proteins has been identified, which contain small MutS-related domains at the carboxy-terminal end [8, 9].

P-type PPRs are considered to participate in group II introns splicing, RNA stabilization, cleavage, translational activation, and transcript accumulation. In contrast, PLS-type PPRs play essential roles in the conversion of cytidine (C) to uridine (U) at specific sites of organelle transcripts [4, 10, 11]. Most of the plant PPR proteins are targeted to mitochondria, chloroplasts, or both, and regulate the functions and

development of these organelles [10]. In the mitochondria, the oxidative phosphorylation system is comprised of five complexes (I–V) [12]. Typical assembly of these complexes is essential to maintain mitochondrial function, which requires the standard processing of mitochondrial pre-mRNAs, including RNA editing and intron splicing [13, 14]. Numerous PPRs are responsible for RNA post-transcriptional processes in mitochondria [15-21].

The E-subgroup PPR proteins (e.g., slow growth1 (SLO1), organelle transcript processing 87 (OTP87), mitochondrial editing factor 3 (MEF3), MEF9, MEF12, and mitochondrial PPR25 (MPR25)) play vital roles in mitochondrial RNA editing and plant development [22-27]. Besides, a few E-subgroup proteins in *Arabidopsis* and rice are also implicated in RNA splicing [28, 29]. In maize, five E-subgroup PPR proteins have been characterized, and all of them are involved in RNA editing. SMALL KERNEL 1 (SMK1) is critical for *nad7*-836 editing in mitochondria, conserved in maize and rice [14]. SMK4 is critical for RNA editing of *cytochrome c oxidase1* (*cox1*) at position +1489 bp [30]. *ccmF*$_N$ is essential for cytochrome c maturation, and the EMPTY PERICARP 7 (EMP7) is responsible for its editing at +1553 bp position. [31]. DEK39 is necessary for RNA editing of the *nad3* transcript in mitochondria [32]. Dek10 is responsible for RNA editing of three sites of the *nad3* and *cox2* transcripts [33]. However, it is still unclear whether E-subgroup PPRs are involved in RNA editing and intron splicing in maize organelles.

Here, we identified the maize mutant *dek55* with an embryo-lethal phenotype with arrested endosperm development, which is caused by the mutation of the mitochondria-localized E-subgroup PPR protein DEK55. In the *dek55* mutant, the splicing efficiency of *nad1* intron 1 and intron 4 *trans*-splicing and *nad4* intron 1 *cis*-splicing were decreased. Moreover, the editing ratios of 24 editing sites (*atp1*-1490, *atp8*-123, *ccmFc*-160, *ccmFc*-799, *ccmFc*-866, *ccmFc*-906, *ccmFc*-1144, *ccmFc*-1244, *ccmFn*-287, *ccmFn*-302, *cob*-564, *mat-r*-1877, *nad3*-146, *nad3*-190, *nad4*-77, *nad6*-25, *nad6*-138, *nad6*-146, *nad6*-159, *nad6*-161, *rps12*-*ct*-418, *rps12*-284, *rps13*-56, and *rps3*-69) were also significantly reduced. Further, DEK55 can interact with ZmMORF1 and ZmMORF8 in yeast, which might be responsible for the action of DEK55 on multiple editing sites. Taken together, our results suggest that the E-subgroup PPR protein DEK55 participates in both RNA editing and group II intron splicing in maize mitochondria.

Results

Genetic and phenotypic analysis of the *dek55-1* mutant

A mutant with a defective kernel phenotype was isolated from an ethyl methane sulfonate-induced maize B73 background population and was named *defective kernel 55-1* (*dek55-1*). The *dek55-1* kernels were segregated from self-pollinated progenies of *dek55-1/+* heterozygotes in a 1:3 mendelian ratio (Fig. 1a, Additional file 1: Table S1). These results suggested that *dek55-1* as a recessive phenotype is caused by a monogenic mutation, which was confirmed in other populations generated from *dek55-1/+* heterozygotes crossed with the inbred lines C733 or S162 (Additional file 1: Table S1).

The *dek55-1* kernels were of smaller size with whitish pericarp and can be distinguished from the wild-type (WT) kernel at 15 days after pollination (DAP) (Fig. 1a). At the maturity, *dek55-1* kernels became even much smaller and shriveled (Fig. 1b, c). To further dissect the mutant phenotype, both WT and *dek55-1* kernels were longitudinally sliced at 15 DAP. As compared to the WT, the mutant kernel has a tiny sized soft endosperm. (Fig. 1d, e). Furthermore, *dek55-1* exhibited a smaller mature embryo and a decreased proportion of hard endosperm compared to WT (Fig. 1f, g). In addition, the kernel weight of *dek55-1* was reduced by approximately 70% compared to that of WT kernels (Fig. 1h). No seed germinated (0/100) was recorded in the field conditions implying that embryo arrest is lethal in the *dek55-1* mutants.

To further investigate the developmental structure of *dek55-1* kernels, we examined the kernel tissue structure of WT and *dek55-1* mutants at 12 and 18 DAP (Fig. 1i–l). At 12 DAP, the *dek55-1* embryo had only a small scutellum arrested at the coleoptile stage and a large interspace between the endosperm the seed coat. In contrast, the WT embryo contained visible coleoptile, shoot apical meristem, scutellum, two leaf primordia, and the kernel was filled with endosperm cells (Fig. 1i, j). At 18 DAP, the WT embryo had developed into complete structures containing four-leaf primordia, shoot apical meristem, and a clearly seen root apical meristem (Fig. 1k), while *dek55-1* embryos only generated one leaf primordium (Fig. 1l). Fewer starch grains were accumulated in *dek55-1* than in WT endosperm cells at this stage (Fig. 1k, l). In addition, a cavity was observed in the *dek55-1* endosperm (Fig. 1l). These results indicate that developmental defects in the embryo and endosperm are present in *dek55-1* mutants.

Map-based cloning of *DEK55*

To identify the *DEK55* gene, we performed the classical map-based cloning strategy to detect F_2 mutant kernels, which were segregated from a self-pollinated filial 1 (F_1) hybrid ear. Four genomic DNA pools (10 mutant kernels per pool), and both of the parents were used to identify the chromosome location of the *DEK55* gene. The six simple sequence repeat (SSR) markers on chromosome 5 were highly correlated with defective kernel phenotypes, implying that the candidate gene may be on chromosome 5. Further analysis showed that the *DEK55* gene is located between umc1705 and umc2302 on chromosome 5 (Fig. 2a). One thousand eight hundred and sixty eight mutant kernels from the F_2 population were genotyped to narrow down the gene location, using six polymorphic molecular markers. Finally, the *DEK55* gene was located on an approximately 1.29 Mb region between the molecular labels M3 and M4 (Fig. 2a). There are 25 putative protein-coding genes in this region (http://ensembl.gramene.org/Zea_mays/Info/Index). To identify the mutated genes, the genomic DNA of 25 candidate genes was amplified and sequenced. Sequence alignment identified a single nucleotide polymorphism in the E-subgroup PPR protein gene (Zm00001d014471). In the *dek55-1* mutant, nucleotide C was replaced by the nucleotide T at +449 bp, resulting in the substitution of amino acid Ser with Phe. However, no change in the mRNA expression level was observed. (Fig. 2a-d). To validate our results, we obtained a new mutant, *dek55-2*, from the maize ethyl methane sulfonate-induced mutant database [34]. The *dek55-2* mutant showed a single nucleotide mutation (G to A) at +729 bp (Fig. 2b), which leads to protein truncation (Fig. 2d). The mutant *dek55-2* also exhibited defective kernels with small and white pericarps (Fig. 2e). The allelic test between

dek55-1 and *dek55-2* heterozygotes revealed that normal and mutant kernels were segregated with the expected 3:1 ratio (normal/mutant; 450/143; P=0.62) in the F_1 ear (Fig. 2e). As a control, all the kernels from the ear that were crossed between the *dek55-2* heterozygote and WT were normal (Fig. 2e). These results indicate that the mutation in PPR gene Zm00001d014471 was responsible for the defective kernel phenotype, so the annotated gene was designated *DEK55*.

DEK55 is a mitochondrial E-subgroup PPR protein

Sequence alignment demonstrated that the *DEK55* gene is 1893 bp long ORF with no introns. *DEK55* encodes a 630 amino acid residue protein containing 13 PPR motifs and an E domain at the carboxy-terminal end (Fig. 2b-d and Additional file 1: Fig. S1). Mutated sites in *dek55-1* and *dek55-2* were located in the third and fifth PPR motifs, respectively (Fig. 2d). The mutation in *dek55-2* resulted in a truncated DEK55 protein missing the last eight PPR motifs and the E domain.

To examine the subcellular localization of DEK55, the *p35S*:DEK55-EGFP vector was constructed and transformed into maize protoplasts. The fluorescence signal of DEK55-EGFP overlapped with Mito Tracker (mitochondria-specific dye) (Fig. 3a), suggesting that in maize, DEK55 is a mitochondrial PPR protein (Fig. 3a). In addition, *DEK55* expression analysis in various maize tissues demonstrated that *DEK55* is relatively highly expressed in root, anther, and ear, with relatively low expression in stem, leaf, silk, tassel, and kernel (Fig. 3b).

DEK55 is involved in the C-to-U editing of 14 transcripts at multiple sites

Usually, PPR proteins take part in modifying organelle transcripts [10]. It has been reported that E-subgroup PPRs participate in the C-to-U editing of mitochondrial pre-mRNA [14, 32, 33]. To explore whether DEK55 is involved in this processing, the transcriptional levels of 35 maize mitochondrial genes encoding functional proteins were analyzed in WT and *dek55-1*. RNA editing of these transcripts in *dek55* (*dek55-1* and *dek55-2*) and WT (WT-1 and WT-2) were detected by the strand- and transcript-specific RNA-seq (STS-PCRseq) strategy [35]. The sequence reads were mapped to the 35 mitochondrial gene transcripts and examined 482 C-to-U RNA editing sites in WT and *dek55* (Additional file 2: Table S1). Compared with the editing ratio of these RNA editing sites between WT and *dek55* (Additional file 2: Table S2), the results revealed that the C-to-U editing ratio of 31 editing sites in the 14 transcripts (*atp1*, *atp8*, *ccmFc*, *ccmFn*, *cob*, *mat-r*, *nad3*, *nad4*, *nad6*, *nad7*, *rps12-ct*, *rps12*, *rps13*, and *rps3*) were significantly altered in *dek55* (Fig. 4, Additional file 2: Tables S2-S4), the editing ratio of 24 sites was decreased (Fig. 4a) and that of seven sites was increased in *dek55* mutants compared with WT (Fig. 4b, Additional file 2: Tables S2, S4). The editing efficiency at the *atp1*-1490, *ccmFn*-287, *mat-r*-1877, and *rps13*-56 sites was dramatically decreased in *dek55-1* and *dek55-2* kernels, and the editing ratio in the *dek55* mutant was more than 50% lower than that in the WT (Fig. 4a, Additional file 2: Table S3). Directed sequencing of RT-PCR products to evaluate the editing efficiency of *atp1*-1490, *ccmFn*-287, *mat-r*-1877 and *rps13*-56 sites also indicated that this was significantly reduced in *dek55* at these RNA editing sites (Fig. 4c). The deficient C-to-U RNA editing leads to altered amino acid residues in *dek55* (Fig. 4c). Meanwhile, at the

atp8-123 site, only the editing efficiency of *dek55-2* (5%) was more than 50% lower than the WT, and at *nad4*-77 sites, only the editing efficiency of *dek55-1* (24.2%) was more than 50% lower than the WT (Fig. 4a). The above results indicated that DEK55 is required for RNA editing at multiple editing sites, especially the *atp1*-1490, *ccmFn*-287, *mat-r*-1877, and *rps13*-56 sites.

DEK55 is essential for the *trans*-splicing of *nad1* introns 1 and 4 and the *cis*-splicing of *nad4* intron 1

The transcript levels of 35 maize mitochondrial genes were examined, and the results depicted that *nad1* and *nad4* were significantly downregulated in the *dek55* mutant (Fig. 5a). The genomic DNA of *nad1* contains four group II introns, and except the 2nd intron, all are *trans*-splicing introns. (Fig. 5c). The genomic DNA of *nad4* has three *cis*-splicing introns (Fig. 5d) [13, 36]. The full maturation of *nad1* and *nad4* transcripts requires complete intron splicing. We further analyze the intron splicing efficiency of *nad1*, *nad4,* and other genes in *dek55-1* and WT by qRT-PCR. Compared with that in the WT, the splicing efficiency of the first and fourth introns of *nad1* and the first intron of *nad4* in the *dek55-1* mutant were decreased (Fig. 5b). Furthermore, we amplified each intron and full transcripts of *nad1* and *nad4* by RT-PCR (Fig. 5c, d). The transcriptional abundance of *nad1* exon 1-2, exon 4-5, and the full-length DNA fragments were significantly decreased (Fig. 5c). RT-PCR could not amplify the intronic DNA fragments (1F+2R, 3F+4R, 4F+5R) in *dek55* and WT because the 1st, 3rd, and the 4th intron of *nad1* were too long. (Fig. 5c). The unspliced 2nd intronic fragments of *nad1* in the *dek55* mutants were similar to those in WT (Fig. 5c). The abundance of *nad4* spliced exon 1-2 and full-length DNA fragments were significantly decreased, and the abundance of the *nad4* unspliced intron 1 transcript was significantly increased (Fig. 5d). Our findings suggest that the significant decrease in the *nad4* and *nad1* transcript abundance in *dek55* mutants was caused by the abnormal splicing of *nad4* intron 1, *nad1* intron 1, and intron 4, respectively (Fig. 5a–d). Therefore, DEK55 is necessary for the *trans*-splicing of the two *nad1* introns (1st and 4th) and *cis*-splicing of the first *nad4* intron in maize.

dek55-1 mutant exhibits reduced complex I activity and increased alternative respiratory pathway activity

The four genes, i.e., *nad1*, *nad4*, *nad3,* and *nad6* encode the subunits of complex I NAD1, NAD4, NAD3, and NAD6, respectively [36]. The *rps13* gene encodes a ribosomal protein, *atp1* and *atp8* encode the ATPase subunit 1 and subunit 8 subunit of ATP synthase F1, respectively [36]. Defects in the post-transcriptional processing of these genes may impair the biosynthesis of mitochondrial complexes [17, 37-39]. We performed blue native polyacrylamide gel electrophoresis (BN-PAGE) and the in-gel NADH dehydrogenase activity assay to investigate the accumulation level and activity of mitochondrial complexes in WT and *dek55*-1 endosperm. BN-PAGE showed that the abundance of complex I and super-complex I+III$_2$ in *dek55-1* mutants was significantly decreased (Fig. 6a). However, no significant differences were observed for the complex V between WT and *dek55-1* (Fig. 6a). Furthermore, the activity of the complex I and I+III$_2$ was reduced in the *dek55-1* mutant (Fig. 6b). These results indicate that defects in mitochondrial transcript splicing and/or editing might affect the abundance and activity of mitochondrial complex I.

The mitochondrial respiratory chain in plants contains the cytochrome *c* and alternative oxidase (AOX) pathways [40]. When the main cytochrome *c* pathway is blocked, AOX activity can be increased to compensate respiration pathways [41]. In *dek55-1*, the functions of complex I were abolished (Fig. 6a, b). Thus, we performed qRT-PCR to detect the expression levels of *Aox* genes in WT and *dek55-1*, and the results showed a 512-fold increase in the expression level of the *Aox2* gene in the *dek55-1* mutant as compared to the WT. (Fig. 6c). Collectively, our results indicate that the respiration pathway is severely blocked in *dek55-1* mitochondria.

DEK55 can interact with ZmMORF1 and ZmMORF8 in yeast

Previous studies explained that the MORFs directly interact with PPR proteins and play a role in RNA editing at numerous editing sites [42, 43]. In this study, DEK55 is responsible for 31 RNA editing in maize, so we speculated that DEK55 might interact with MORFs to form an editing complex involved in RNA editing in maize. Thus, we used MORFs in *Arabidopsis* as baits to search for putative MORFs in maize. Seven putative MORFs were identified in maize (Fig.7a). A yeast two-hybrid assay was performed to screen for MORFs interacting with DEK55, and the results indicated that the DEK55 can interact with ZmMORF1 and ZmMORF8 in yeast (Fig. 7b).

Discussion

DEK55 is required for maize kernel development

Previous reports have shown that PPR proteins play vital roles in maize kernel development and the loss of function of some PPR proteins results in empty pericarp and small and defective kernel phenotype in different genetic backgrounds [13, 14, 17-21, 30, 31, 39, 44, 45]. The *ppr* mutants exhibit developmentally arrested embryos and endosperm. The embryos usually reached the coleoptile stage or the leaf stage 1 (L1), and the endosperm exhibited significantly reduced starch and protein levels [14, 33, 45]. The *dek55* mutant showed small size kernels with shriveled pericarp (Fig. 1a-c). Moreover, the mutant kernels had smaller embryo and a decreased proportion of hard endosperm compared with the WT (Fig. 1d-l). In particular, the *dek55-1* embryo was severely arrested and had only one leaf primordium. Thus, the mutant kernel would not be able to germinate in the field. Allelic tests indicated a nonsense mutant *dek55-2*, an allelic mutant with *dek55-1* and *dek55-1/dek55-2* heterozygous kernels, displayed a phenotype similar to *dek55-1*. This suggests that *dek55* dysfunction is responsible for the defective kernel phenotype and that DEK55 is required for kernel development in maize.

The E-subgroup PPR proteins are characterized by an E domain at the carboxy-terminal end that might be responsible for interactions between proteins [46, 47]. In the *dek55-1* mutant, there is a single nucleotide change (C to T) at +449 in *dek55*, which caused phenylalanine (Phe) to replace serine (Ser) on the third PPR motif of DEK55 at position 150 in the polypeptide chain (Fig. 2b, d; Additional file 1: Fig. S1). This mutation from a hydrophilic to a hydrophobic amino acid would alter the affinity of the protein to water. Our evidence suggests that the amino acid change (Ser→Phe) is responsible for defective kernels in *dek55* mutants. Therefore, the amino acid change at this site in DEK55 might cause conformational

changes and loss of function. In *dek55-2* mutants, the mutation resulted in a loss of the last eight PPR motifs and E domain at the carboxy-terminal end of the DEK55 protein (Fig. 2b, d), which might prevent it from forming complexes with other proteins and binding to the targets sites.

DEK55 is necessary for C-to-U editing of multiple sites in the mitochondrial transcripts of maize

PPR proteins, including DYW2, EMP21, NUWA, MEF8, and DEK53, are involved in C-to-U editing at multiple sites [48-52]. In this study, we demonstrated that DEK55 participated in RNA editing at 31 sites, while the editing ratio of 24 editing sites was reduced and that of seven editing sites was increased in *dek55*, suggesting that DEK55 is also necessary for RNA editing at multiple sites. Among them, DYW2 and MEF8 harbor only five PPR repeats and belong to an atypical DYW subgroup [48, 49]. NUWA belongs to the P-class of PPR proteins [48, 50]. EMP21 contains 11 PPR-motifs in addition to E and DYW domains and belongs to PPR-DYW proteins [51]. DEK53 is an E-subgroup PPR protein with seven PPR repeats [52]. DEK55 is considered as an E-subgroup PPR protein that contains the canonical E domain. Therefore, PPR proteins that target multiple sites for editing might not share similar structures.

MORFs can interact directly with PPR proteins to participating in RNA editing [42, 43, 51]. In *Arabidopsis*, MEF13 (an E-subgroup PPR protein) interact with MORF3 and MORF8. The protein complex is responsible for RNA editing of the same sites between *morf3*, *morf8,* and *mef13* mutants [42]. EMP21 is necessary for the editing of ~17% of mitochondrial target Cs in maize [51].Interestingly, 34 editing sites overlap in maize *emp21* mutants and *Arabidopsis morf8* mutants, and eight editing sites overlap in maize *emp5* mutants and *morf8* mutants. ZmMORF8 (GRMZM2G169384), an ortholog of MORF8 in maize, directly interacts with EMP21 and EMP5, suggesting that EMP21 and EMP5 participate in the editing of some sites by interacting with ZmMORF8 [51]. DEK53 is an E-subgroup PPR protein that interacts with ZmMORF1 to form an RNA editing complex, and which is responsible for more than 60 RNA editing in the maize mitochondrion [52]. In our study, DEK55 participated in C-to-U editing of 14 mitochondrial transcripts at 31 editing sites, while the editing ratios of 24 sites were decreased in *dek55* (Fig. 4a, Additional file 2: Table S3) (Fig. 4). Meanwhile, comparative analyses of these mitochondrial transcript C-to-U editing events in both *Arabidopsis* and maize indicated that the multiple sites were not edited in *Arabidopsis*, as these sites are “Ts”, e.g., *ccmFc*-799, *ccmFc*-866, *ccmFc*-1144, *ccmFc*-1244, *cob*-564, *mat-r*-1877, *nad3*-190, *nad4*-77, and *nad6*-146. This suggests that editing in these sites may is necessary in maize. In addition, editing was also substantially impaired in *morf8* mutants of *Arabidopsis* at the following four sites: *atp1*-1484 (*atp1*-1490 in *dek55* mutant), *ccmFc*-160, *nad6*-161, and *rps12*-284[35]. DEK55 can interact with ZmMORF8 (ortholog of AtMORF8 in maize), shown by yeast two-hybrid assay. Although the *Zmmorf8* mutant has not been identified in maize yet, our findings might provide evidence that the DEK55 might interact with ZmMORF8 to function at these RNA editing sites in maize. In *dek53*, over 60 RNA editing sites were affected [52], and three of them (*nad3*-146, *nad6*-146, and *atp8*-123) were also affected in *dek55*. DEK53 and DEK55 interact with ZmMORF1 [52, this study]. So, the DEK53 and DEK55 might be responsible to C-to-U RNA editing of these sites through interaction with ZmMORF1. These results indicate that ZmMORF8 and ZmMORF1 might interact with DEK55 to form an editing complex for these multiple editing sites.

DEK55 is involved in group II intron splicing in maize mitochondria

The E-subgroup PPR proteins are considered to be editing factors for RNA editing in organelles [10], whereas few of these proteins are considered to play a role in splicing [28, 29, 53]. SLO4 is necessary for RNA editing of *nad4* and the efficient splicing of *nad2* intron 1 in *Arabidopsis* mitochondria [29]. AEF1/MPR25 participates in RNA editing of *atpF* and *nad5* and modulates *atpF* splicing in both *Arabidopsis* and rice [28]. Furthermore, the plastid PPR protein OTP70 only participates in the intron splicing of the *rpoC1* transcript [53]. In this study, DEK55 (an E-subgroup PPR protein) participated in both RNA editing of 31 sites and group II intron splicing in maize mitochondrial transcripts (Figs. 4, 5a-d). The RNA editing ratio of the *nad1* transcripts was not affected, and only *nad4-77* was reduced in *nad4* transcripts. Meanwhile, it has been reported that intron splicing is usually mediated by RNA editing events in which the key sites of introns are edited [54-56]. The editing efficiency of two RNA editing sites (147 and 409) on the *nad4* intron 1 was increased in *dek55* (Additional file 2: Table S1), which might result from the reduction in the splicing efficiency of the *nad4* intron 1. The editing efficiency of the RNA editing sites on *nad1* introns 1 and 4 could not be analyzed because the introns were too long to amplify. Thus, it could not be confirmed whether the decreased splicing efficiency of *nad1* introns 1 and 4 in *dek55* was caused by editing events in the intron.

Several proteins have been identified that participate in the splicing of *nad1* and *nad4* pre-mRNAs. The nuclear maturases 1 [57], DEK2 [45], and EMP11 [44] participate in *trans*-splicing of *nad1* intron 1. EMP11, EMP8, and ZmSMK3 are required for *nad1* intron 4 *trans*-splicing [44]. The proteins include NMS1 [58], DEK35 [19], EMP8 [13], DEK43 [20], EMP602 [59], and ZmSMK3 [60] and are implicated in *cis*-splicing of *nad4* intron 1. In our study, we have demonstrated that DEK55 is involved in both *trans*- and *cis*-splicing. It appears that splicing of one intron may require the involvement of multiple factors to constitute a putative spliceosome. This is supported by the finding that PPR-small MutS-related-1 can interact with the Zm-mCSF1 to form protein complex. This protein complex participates in the intron splicing of multiple transcripts within the mitochondria [61]. Therefore, DEK55 might interact with P-type PPR proteins or other splicing factors involved in group II intron splicing.

Conclusions

In this study, we have demonstrated that DEK55 is a mitochondria-localized E-subgroup PPR protein. Mutation of DEK55 leads to embryo-lethal and arrested endosperm development phenotype in maize. DEK55 is required for editing at 31 RNA editing sites, especially the atp1-1490, ccmFn-287, mat-r-1877, and rps13-56 sites (Fig. 4). Meanwhile, DEK55 can interact with ZmMORF1 and ZmMORF8 in yeast. In addition, DEK55 is responsible for the trans-splicing of two nad1 introns (intron 1 and intron 4) and cis-splicing of the nad4 intron 1 in mitochondria. Our results suggest that the E-subgroup PPR protein DEK55 plays important roles in the RNA editing and the splicing of introns of maize mitochondrial transcripts. These results provide a novel view for understanding the molecular function of E-subgroup PPR proteins in RNA processing in plant organelles.

Methods

Plant materials

Maize mutant *dek55-1* identified from ethyl methanesulfonate populations having a B73 background was kindly provided by Prof. Xiaoduo Lu of Qilu Normal University. The original name of the allele mutant *dek55-2* is EMS4-073342. The EMS4-073342 was purchased from the maize ethyl methane sulfonate-induced mutant database (http://www.elabcaas.cn/memd/) [34], found by searching for the gene ID (Zm00001d014471). To purify the genetic background, *dek55-1* was back-crossed with the B73 inbred line, and BC_2F_2 kernel was used in this study. The *dek55-1* heterozygote as the male parent was crossed with our laboratory inbred lines C733 and S162, then F_1 progenies were self-pollinated to generate the F_2 population that was used for gene mapping. Ru Chang Ren and Xu Wei Yan undertook the formal identification of the plant materials. All the plant materials were sown at the experimental station of Shandong Agricultural University (Taian, Shandong Province).

Histological analysis

The WT and defective kernels were obtained from the self-pollinated heterozygous plant at 12 and 18 DAP, respectively. The middle part of the kernel along the longitudinal axis was selected and placed in formalin-acetic acid-alcohol solution for at least 12 h on ice, followed by treatment with 50%, 70%, 85%, 95%, and 100% ethanol, and later the seeds were placed in 100% xylene for 2-4 h. After dehydration, materials were immersed in the molten paraffin for 72 h at 60 °C and then embedded in paraffin. The paraffin-embedded samples were cut into 12 μm slices using a microtome (Leica RM2235, Germany). Section staining was performed based on the methods of Ren et al. (2020). Finally, the sections were photographed with a light microscope (Olympus DP72, Olympus Life Sciences, Waltham, MA, USA).

Map-based cloning

The *DEK55* locus was identified using 1868 F_2 defective kernels from the self-pollinated F_1 population (C733×*dek55-1/+*). For preliminary mapping, 73 polymorphic SSR markers, selected from the entire genome, were used to screen the parents, F_1 individual plants, and four groups of pooled DNA of the F_2 defective kernels. For fine mapping, new molecular markers were selected according to the parental DNA sequences. The website (http://ensembl.gramene.org/Zea_mays/Info/Index) was used to search for the genes annotated in the candidate regions of the *Zea mays* genome (B73_RefGen_v4) [62]. Phanta EVO Super-Fidelity DNA polymerase (catalog number P503-d1, Vazyme Biotech Co., Nanjing, China) was used to clone all candidate gene genomic DNA sequences and sequencing. The primers were designed according to the candidate gene reference sequences. Primers used to clone the full-length *DEK55* gene and map-based cloning are given in Additional file 1: Table S2.

RNA extraction, RT-PCR, and qRT-PCR

Total RNA from WT and *dek55* mutant kernels without pericarp and other tissues were extracted with the Ultrapure RNA Kit (CWBIO, China). The residual DNA in the total RNA was removed by DNase. For RT-PCR, complementary DNA (cDNA) was obtained by reverse transcription, and used as the template for polymerase chain reaction (PCR) amplification, the KOD DNA polymerase (KOD FX Neo, code: KFX-201, Toyobo, Japan) was used to PCR. PCR procedures as follows: initial melting at 94°C for 2 min, then by 33 to 38 cycles of 15 s at 98°C, 30 s at the applicable annealing temperature (58°C to 61°C) for the varies primer pairs and adequate extension times (30 s/ per kilobase) at 68°C, then final extension at 68°C for 7 min. RT-PCR was performed to amplify mitochondrial transcripts, splicing efficiency of *nad1,* and *nad4* introns. The DNA fragments obtained by RT-PCR were directly sequenced. The transcripts were amplified according to the primers previously reported [63], and the primers are list in Additional file 1: Table S2. Primers used to amplify introns of *nad1* and *nad4* are shown in Additional file 1: Table S2.

The qRT-PCR equipment and reaction system were used according to a previous report [20]. All qRT-PCR assays were performed with three samples and technical repeats. The primers were designed for group II intron splicing efficiency analysis in mitochondria according to previous reports [17, 18, 63]. The primers used to analyze the *DEK55* expression levels are shown in Additional file 1: Table S2.

RNA editing efficiency analysis

The RNA editing of 35 mitochondrial genes were analyzed by the Strand- and transcript-specific RNA-seq (STS-PCRseq) method described by Bentolila *et al*., [35] with few modifications. The 35 mitochondrial gene transcripts and *nad4* intron 1 were amplified through RT-PCR using four cDNA libraries as templates. These cDNA libraries were obtained from the WT and *dek55* mutant kernels (WT-1 and *dek55-1*, WT-2 and *dek55-2*), obtained from the self-pollinated *dek55-1/+* and *dek55-2/+* heterozygote ears at 15 DAP. The PCR products were detected on 1% agarose gels; the gel bands were excised, and the DNA fragments were purified using the Gel DNA Extraction Mini Kit (Vazyme, Cat. no. DC301-01). Purified DNA samples amplified from the same cDNA library were mixed as equimolar amounts and sheared by sonication to generate 300-500 bp DNA fragments. The DNA library was constructed and sequenced on the Illumina platform by Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). The sequenced data was filtered for the lower quality reads, the adaptor reads, and the unknown base reads. The clean reads were mapped to the 35 mitochondrial gene transcripts and *nad4* intron 1 using 'Bowtie 2', and the allele frequency count was performed as described previously [52]. The editing efficiencies of 482 RNA editing sites were calculated, and the affected editing site in the *dek55* mutant was defined as described by previously reported [51]. Editing efficiency was described as decreased in the *dek55* mutants when the ratio of (T/(T+C)% in *dek55*-T/(T+C)% in WT) was ≤ -10%; while editing efficiency was considered as increased in the *dek55* mutant when the ratio of (T/(T+C)% in *dek55*-T/(T+C)% in WT) was ≥10%. Overlapping sites affected in *dek55-1* vs. WT-1, *dek55-1* vs. WT-2, *dek55-2* vs. WT-1, *dek55-2* vs. WT-2 were considered as affected editing sites [51].

Yeast two-hybrid assay

The full-length ORF of DEK55, excluding the signal peptide coding sequence (1-207 bp), was amplified using specific primers. The PCR products were cloned into the pGBKT7 vector (Clontech, Kyoto, Japan) at *EcoRI* and *BamHI* sites to generate the DEK55-BD bait vector. The coding sequences of seven ZmMORFs were amplified using special primers. Subsequently, the PCR products were cloned into pGADT7 vector (Clontech, Kyoto, Japan) to generate the recombinant ZmMORFs-AD prey vectors. The recombinant vectors of DEK55-BD and ZmMORFs-AD were co-transferred into Y2H Gold competent cells. The empty pGBKT7 and pGADT7 vectors were used as negative controls. The transformed cells were incubated on synthetic dextrose (SD)/-Leu-Trp dropout plates and the SD/-Leu-Trp-His-Ade dropout with X-*α*-gal plates at 30°C for 3 days. The primers used are listed in Additional file 1: Table S2.

Subcellular localization

Complete ORF, excluding the stop codon, of the *DEK55* gene was incorporated into the pM999-EGFP vector generating a DEK55-EGFP recombinant vector driven by the CaMV 35S promoter. Subcellular localization was performed as reported previously [64]. In short, the protoplasts of maize mesophyll cells were obtained from etiolated leaves by enzymatic hydrolysis as described previously [21]. Recombinant vector (20 μL, 15-20 μg) was added into the 200 μL maize protoplast solution, 220 μL of 40% (w/v) PEG4000 solution was added and mixed completely, and then the samples were incubated at 23 °C for 10-15 min. Afterward, the protoplasts were washed using a W5 or WI solution and cultured for 12-16 h in the dark at 23 °C. Before imaging, protoplasts were stained with a mitochondria-specific dye (MitoTracker Red CMXRos, Thermo Fisher Scientific, Waltham, MA, USA), and the samples were observed using a laser confocal microscope (LSM 880, Zeiss, Jena, Germany).

Isolation and analysis of mitochondrial complexes

The plant mitochondrial isolation kit (Biohao, Wuhan China; catalog no. P0045) was used to isolate crude mitochondria from WT and *dek55-1* seed tissue, excluding the pericarps (on 15 DAP) for the BN-PAGE and complex I activity analysis. The collected mitochondrial precipitate was redissolved in 35 μL of solution buffer (50 mmol/L Bis-Tris, 6 N HCl, 50 mmol/L NaCl, 10% w/v glycerol, 0.001% Ponceau S, pH 7.2, containing 20% n-dodecyl-b-D-maltoside (Sigma-Aldrich, St Louis, MI, USA) to a final concentration of 1%) and then kept on ice for 30 min. The suspension was then centrifuged at 4 °C, the supernatant was collected and loaded on pre-prepared gradient gels (BN1002BOX, Thermo Fisher Scientific), and electrophoresis was performed according to the manufacturer's instructions. Next, the gels were placed in 100 mL of fixing solution (methanol/ddH_2O/acetic acid, 4:5:1) for 30 min and then transferred to 0.02% Coomassie R-250 stain (Sigma-Aldrich, St Louis, MI, USA) for mitochondrial-complex abundance analysis. The gel strips were incubated in assay buffer (25 mg nitrotetrazolium blue and 100 μL NADH (10 mg/mL) combined with 10 mL of 5 mmol/L Tris/HCl, pH 7.4) (Sigma-Aldrich) for 5 min, and the reaction was terminated with the fixing solution (40% methanol/10% acetic acid (v/v)) for analysis of complex I activity [44].

Abbreviations

AOX: alternative oxidase; atp1: ATP synthase subunit1; BN-PAGE: blue native polyacrylamide gel electrophoresis; C: cytidine; cox: cytochrome c oxidase; cDNA: complementary DNA; DAP: days after pollination; dek: defective kernel; EGFP: enhanced green fluorescent protein; EMP: Empty pericarp; Leu: Leucine; MEF: mitochondrial editing factor; MORFs: Multiple organellar RNA editing factors; MPR25: mitochondrial PPR25; nad: NADH dehydrogenase; ORF: open reading frame; OTP87: organelle transcript processing 87; PCR: polymerase chain reaction; Phe: Phenylalanine; PPR: Pentatricopeptide repeat; Pro: Proline; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; rps13: ribosomal protein S13; RT-PCR: reverse transcription-polymerase chain reaction; SD: synthetic dextrose; Ser: Serine; SLO1: slow growth1; SMK: Small kernel; SSR: simple sequence repeat; STS-PCRseq: Strand- and transcript-specific RNA-seq; U: uridine; WT: wild type

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The experiments were conceived and supervised by XYZ. RCR, YXW, YJZ, YMW, JZ, JWW, and GMZ performed the experiments. XL isolated the *dek55-1* mutant. The manuscript was drafted by XYZ and RCR, corrected by XHD, and XSZ. All authors read and approved the final manuscript.

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Not applicable.

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Figures

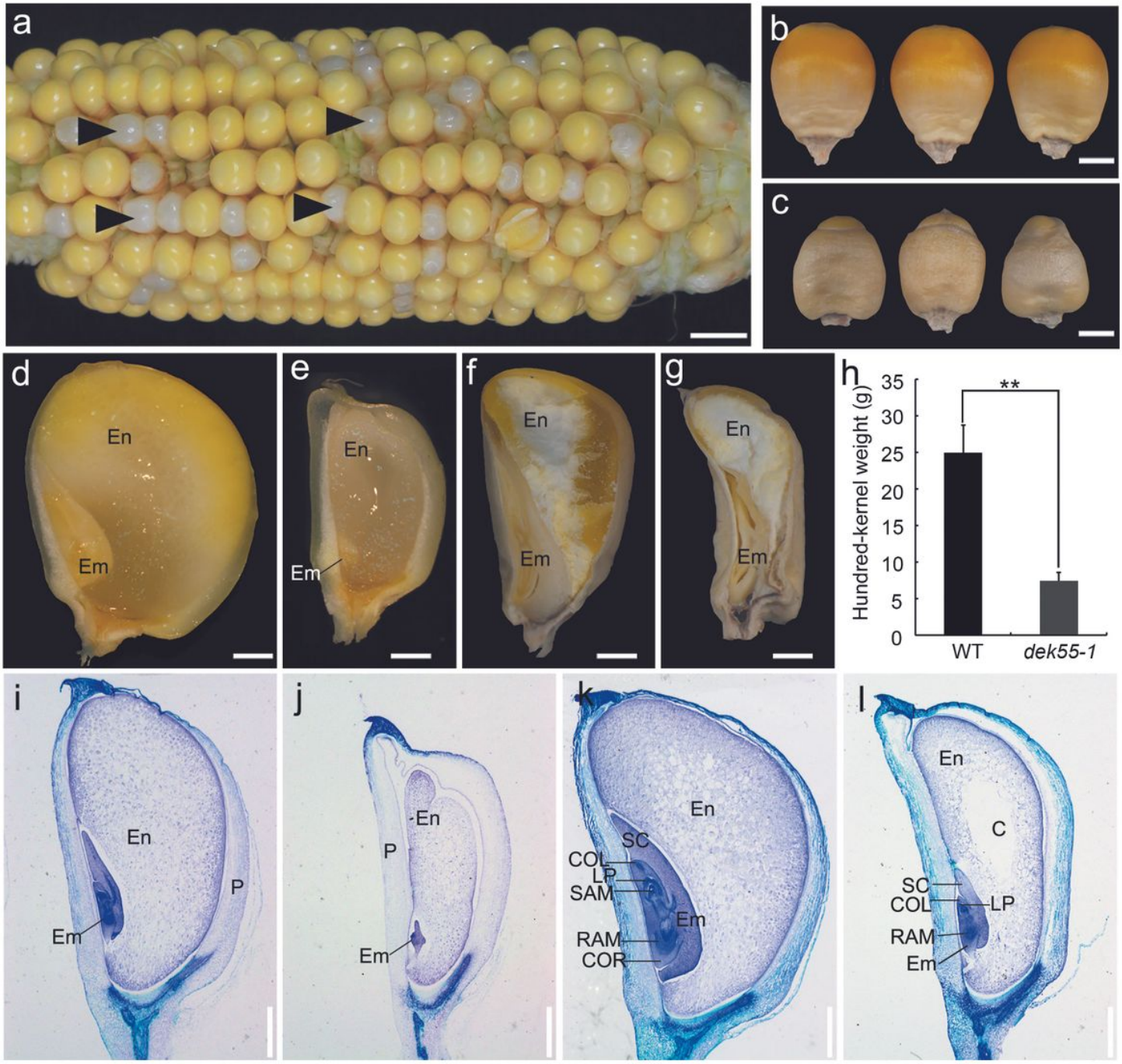


Figure 1

Phenotypic characterization of dek55-1 kernels. (a) The self-pollinated dek55-1 heterozygotes ears at 15 DAP. Some mutant kernels are indicated with arrowheads. Scale bars=1 cm. (b-c). The mature kernels of wild type (WT) and dek55-1. (b), WT; (c), dek55-1. Scale bars=2 mm. (d-g) Comparative anatomy of WT and dek55-1 kernels at 15 DAP and maturity. (d and f), WT kernels. (e and g) dek55-1 kernels. Scale bars=1 mm. (h) Hundred-kernel weight of WT and dek55-1 kernels at maturity. (Asterisks indicate significantly different, **P < 0.05, Student's t-test) (i-l) Histological analysis of WT and dek55-1 kernels at 12 and 18 DAP. (i and k), WT at 12 and 18 DAP. (j and l) dek55-1 kernels at 12 and 18 DAP. Scale bars=1

mm. En, endosperm; Em, embryo; P, pericarp; LP, leaf primordia; RAM, root apical meristem; SAM, shoot apical meristem; SC, scutellum; COL, coleoptile; COR, coleorhiza; C, cavity.

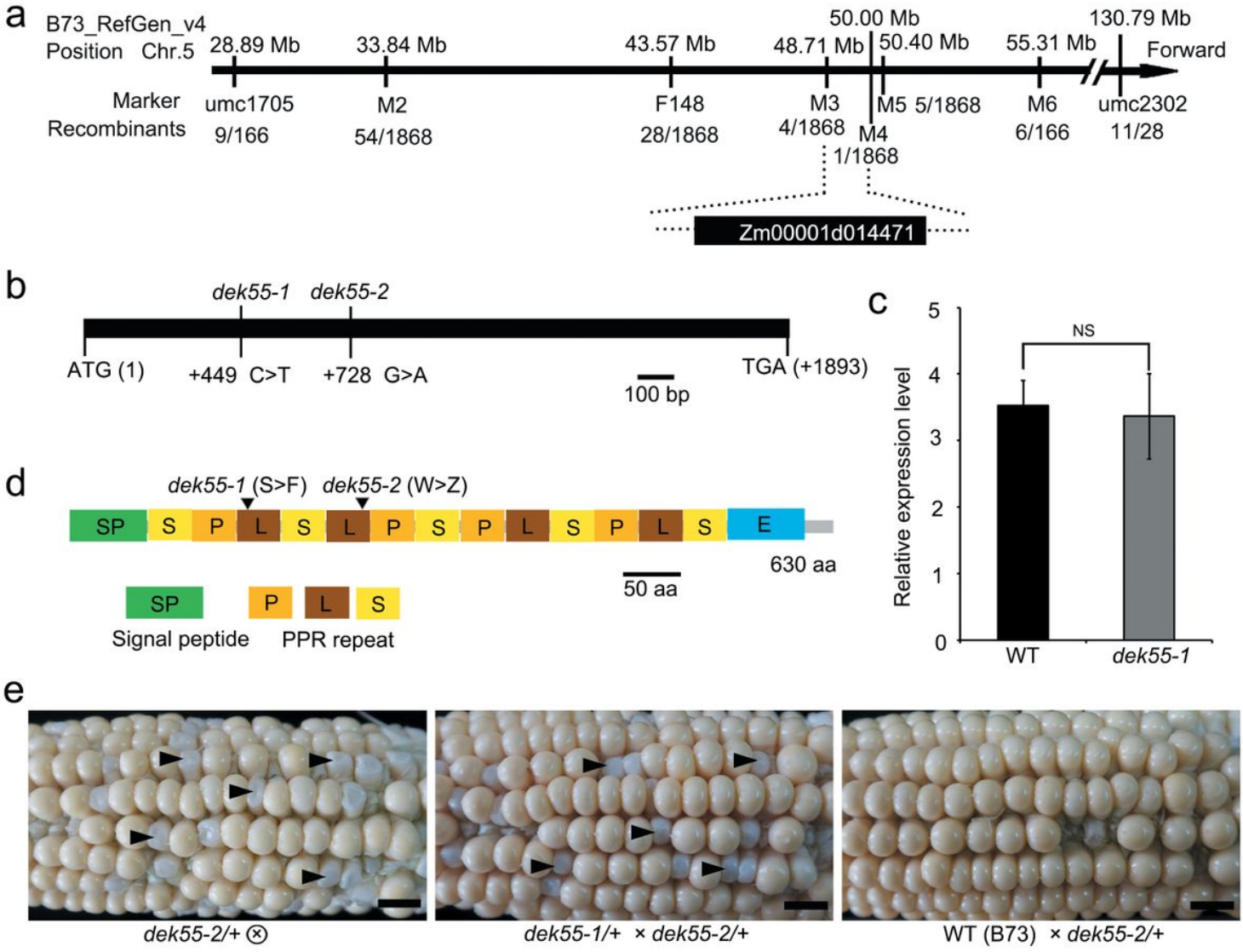


Figure 2

Map-based cloning and identification of DEK55. (a) Fine mapping of the DEK55 locus. The DEK55 locus was mapped to a 1.29 Mb region between marker 3 (M3) and M4 on chromosome 5, in which there are 25 candidate genes. The physical location of polymorphic molecular markers and number of recombinants are shown in the schematic diagram. (b) Schematic structure of dek55 gene. The mutation sites of dek55-1 and dek55-2 are shown. (c) Relative expression level of DEK55 in WT and dek55-1. Values are means of three biological replicates. Error bars represent the standard deviation (SD). (Not significant (NS), $P > 0.05$, Student's t-test). (d) Schematic diagram of DEK55 protein containing total 13 PPR domains (P, L and S) and E domain. The amino acid changes in dek55-1 and dek55-2 are indicated. (e) The self-pollinated dek55-2/+ (heterozygote) at 15DAP, dek55-1/+ and dek55-2/+ were used in an allelism test of dek55. The dek55-2/+ cross to B73 (WT) as control. Some mutant kernels are indicated by black arrowheads. Scale bars=1 cm.

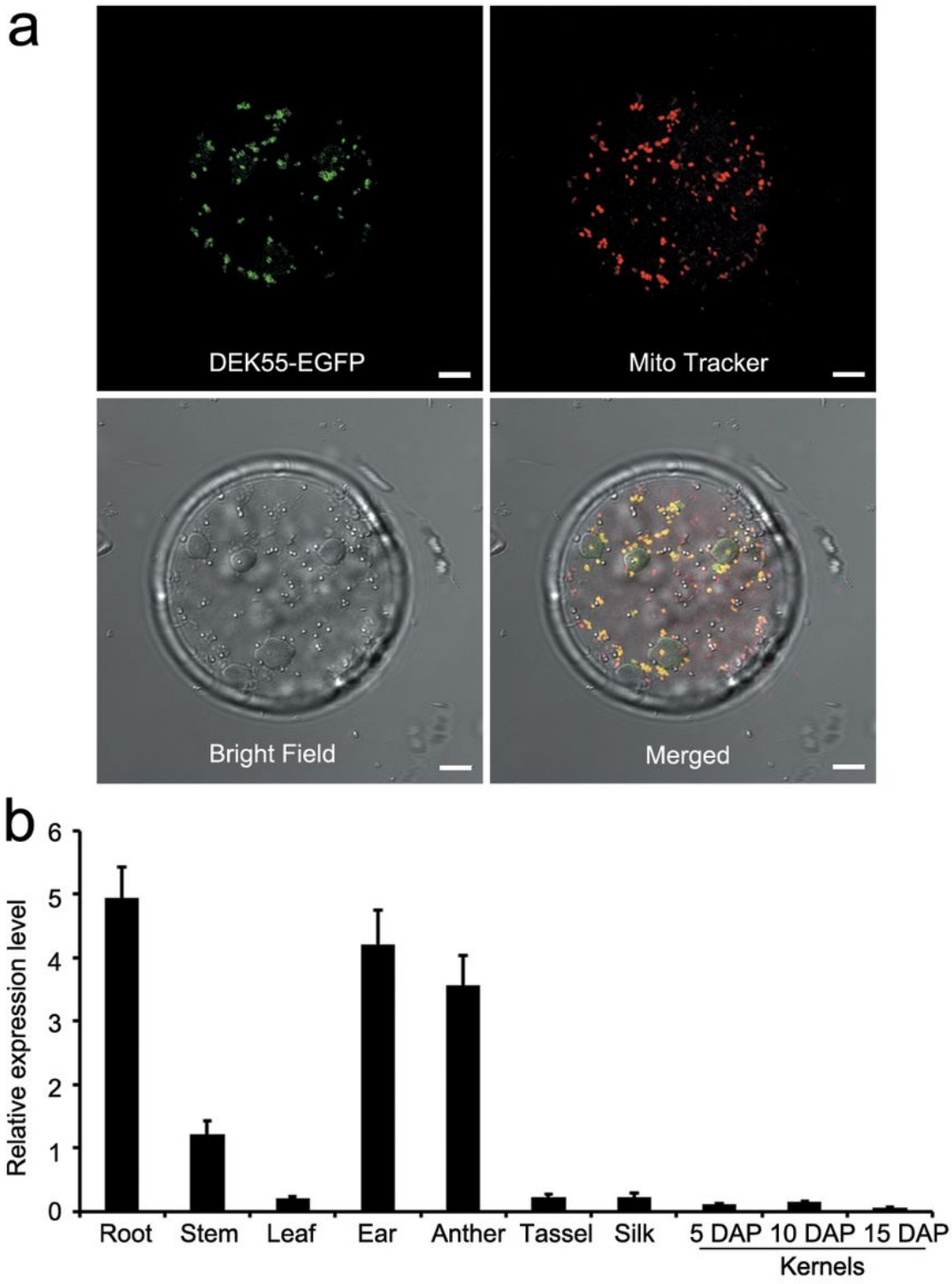


Figure 3

Subcellular localization of DEK55 and expression pattern of DEK55. (a) The subcellular localization of DEK55 was determined by transient expression of DEK55-EGFP fusion protein in maize protoplast. Mitochondria were marked by Mito Tracker (red). Scale bars=5 μm. (b) Analysis of the relative expression level of DEK55 in a various tissues and kernels at 5, 10, and 15 DAP. ZmActin gene (GRMZM2G126010)

was used as an internal control. Values are means of three replicates. Error bars represent the standard deviation (SD).

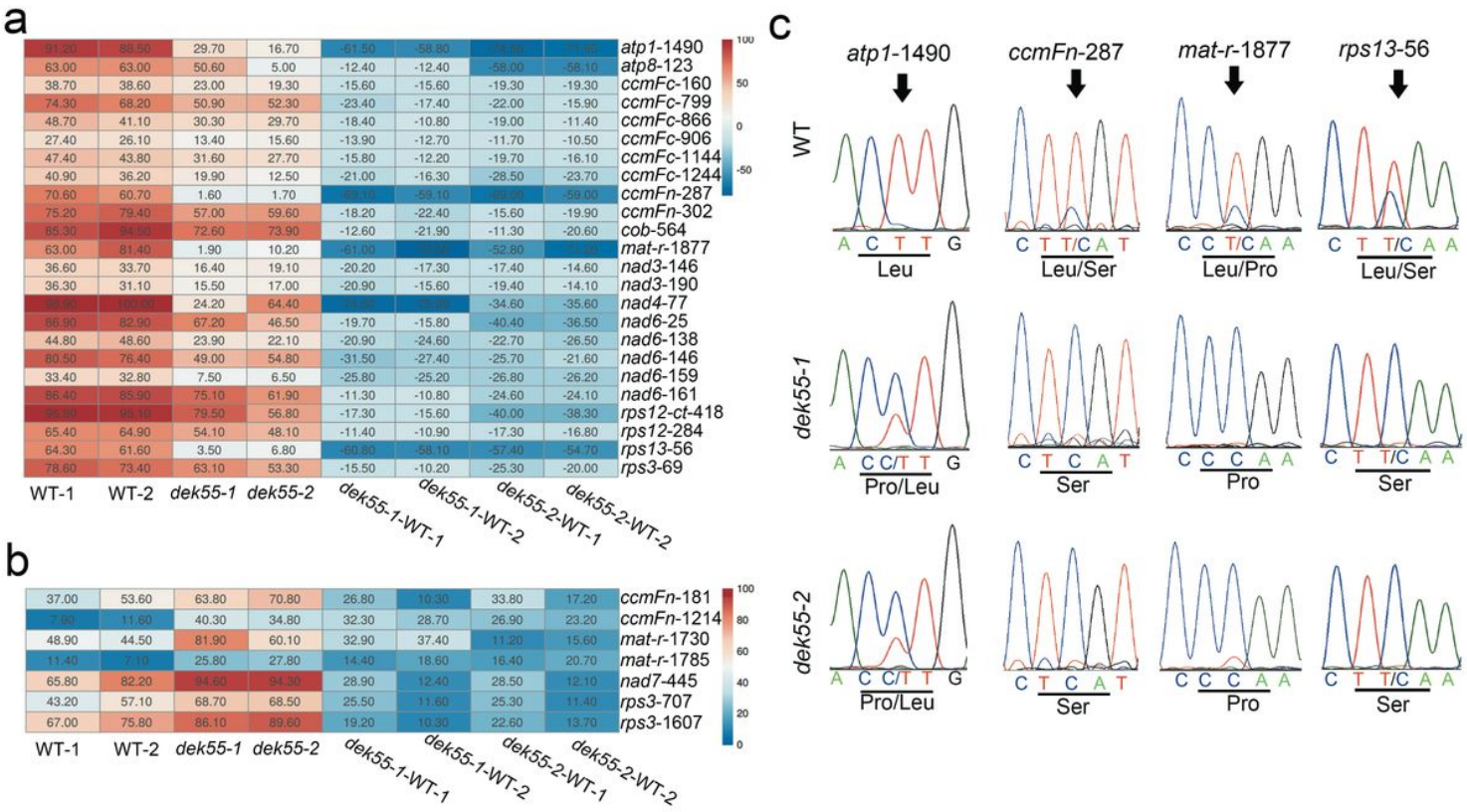


Figure 4

RNA C-to-U editing of 14 mitochondrion transcripts at multiple sites in maize mitochondria (a-b) Heatmaps showing sites where the RNA editing efficiency of dek55 decreased (a) and increased (b) compared to WT, respectively. Editing efficiency of each sites in WT and dek55 are indicated. The variation in editing efficiency of dek55 compared to WT is denoted by dek55-WT. (c) The sequence chromatograms containing the editing sites are shown. Arrows mark the editing sites. The amino acid in the editing site is indicated on the bottom.

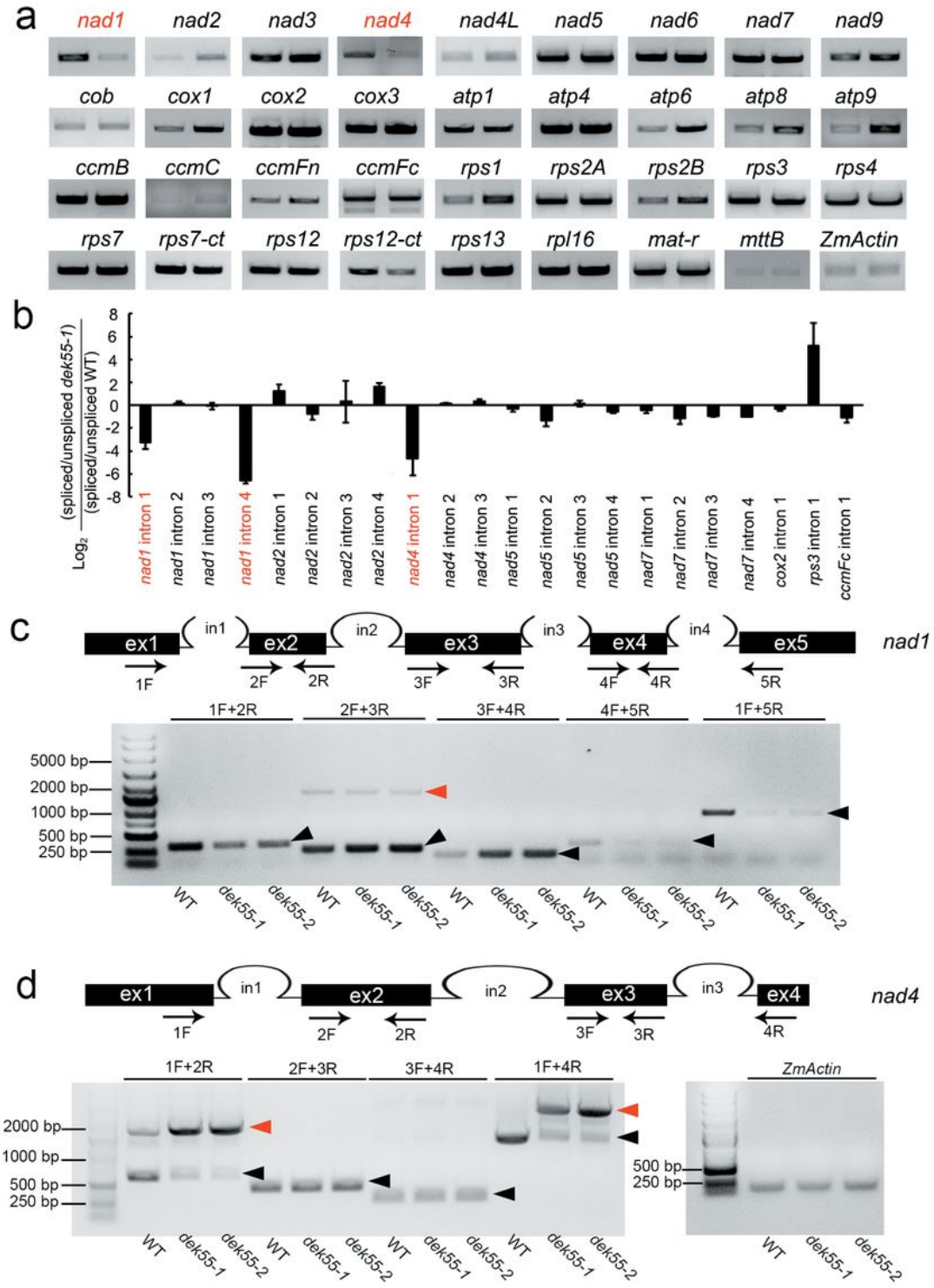
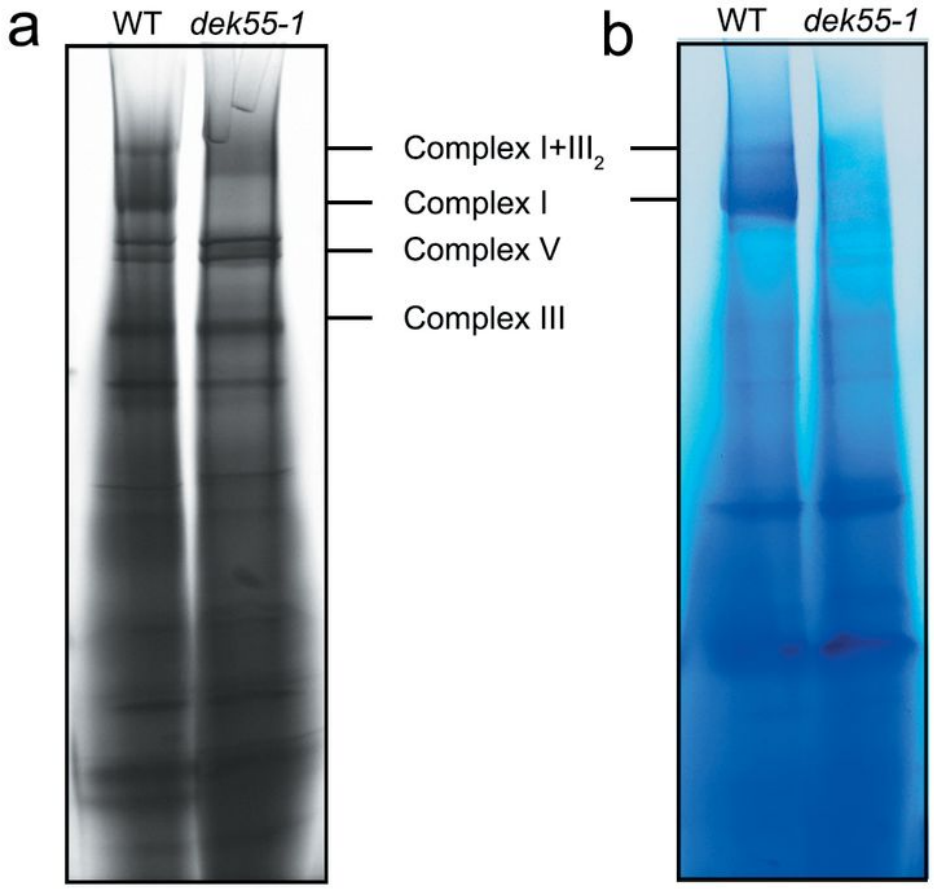


Figure 5

The post-transcriptional RNA processing of nad1 and nad4 was affected in dek55. (a) The expression of 35 mitochondrion-encoded genes in WT (left) and dek55-1 (right) were detected by RT-PCR. ZmActin gene (GRMZM2G126010) was used as an internal control. Both nad1 and nad4 were marked in red because their transcript abundant were significantly decreased. (b) The splicing efficiency of all 22 group II introns in maize mitochondrial-encoded genes was determined in dek55-1 and WT kernels by qRT-PCR. Values

shown are calculated from the mean of three biological replicates, and error bars represent the standard deviation (SD). (c-d) Schematic structure of nad1 gene (c) and nad4 gene (d). The primers used for amplification are indicated. RT-PCR analysis of intron-splicing efficiency of nad1 in WT, dek55-1 and dek55-2 mutant kernels at 15 DAP. All PCR products were confirmed by sequencing. ZmActin gene (GRMZM2G126010) was used as an internal control. The unspliced and spliced fragments are indicated by red and black arrowheads. Exon is indicated as “ex”, and intron is indicated as “in”. The gel images in (a, c, d) were cropped and original gel images are shown in the Additional file 1: Figs. S2-S3.



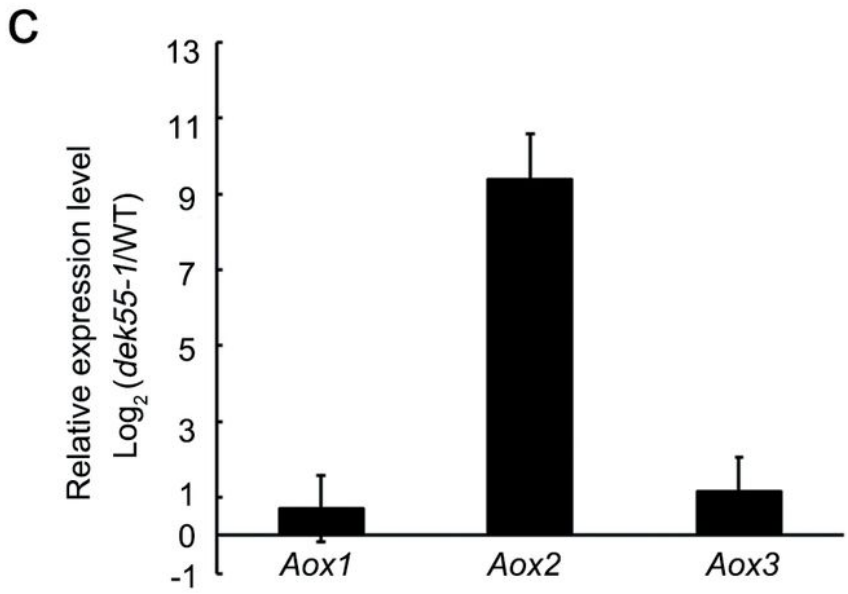


Figure 6

The mitochondrial function was impaired in dek55-1 mutant. (a) BN-PAGE analysis of mitochondrial complexes isolated from WT and dek55-1 kernels at 15 DAP. The gels were stained with Coomassie Brilliant Blue. The positions of the mitochondrial complexes are marked. (b) In-gel NADH dehydrogenase activity analysis of complex I. The positions of complex I and super complex I+III2 are indicated. (c) qRT-PCR analysis of Aox genes (Aox1, Aox2, and Aox3) expression in WT and dek55-1 kernels at 15 DAP. ZmActin gene (GRMZM2G126010) was used as an internal control. Values shown are calculated from the mean of three biological replicates, and error bars represent the standard deviation (SD). The gel images in (a-b) were cropped and the original gel images are shown in the Additional file 1: Fig. S4.

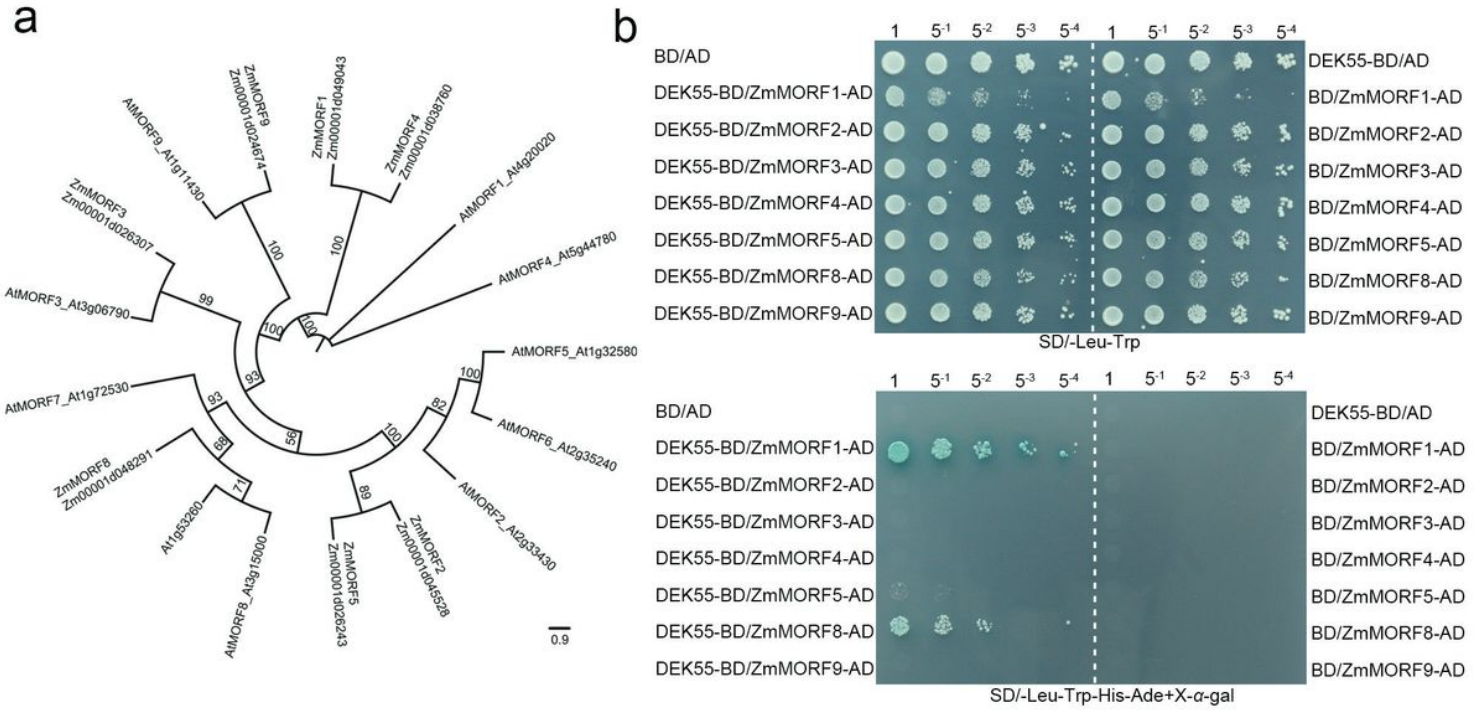


Figure 7

DEK55 can interact with ZmMORF1 and ZmMORF8 in yeast. (a) Phylogenetic tree analysis of known AtMORFs in Arabidopsis and putative ZmMORFs in maize. (b) The yeast two-hybrid assay interaction between DEK55 and ZmMORFs. The different dilution series (1, 5-1, 5-2, 5-3, 5-4) yeast cells were cultured in SD/-Trp-Leu dropout and SD/-Trp-Leu-His-Ade with X-α-gal dropout plates at 30˚C, respectively, with results recorded after three days' culture.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1revision.docx
- Additionalfiles2.xlsx